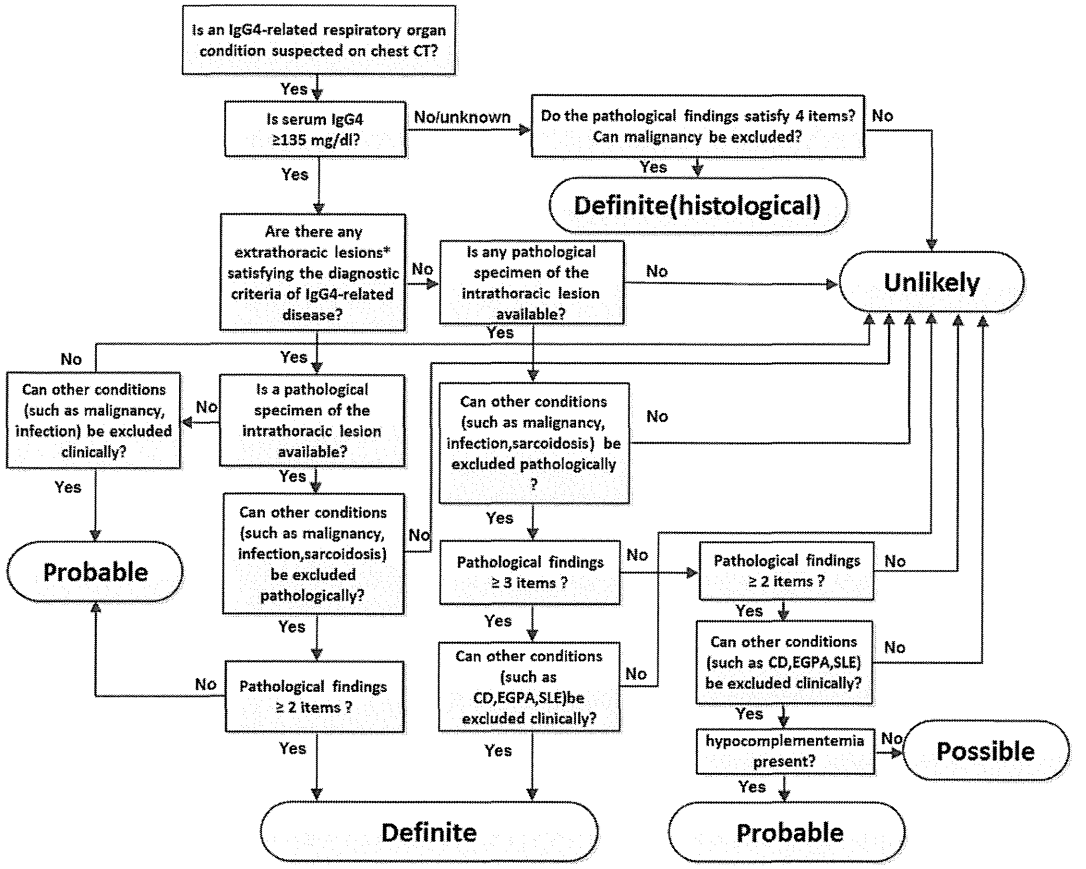

Fig. 1 – Diagnostic algorithm. CD: Castleman disease (plasma cell type) EGPA: eosinophilic granulomatosis with polyangiitis SLE: systemic lupus erythematosus *sclerosing dacryoadenitis· sialadenitis, autoimmune pancreatitis, IgG4-related sclerosing cholangitis, IgG4-related kidney disease, retroperitoneal fibrosis.

Conflict of interest

None of the authors have any relevant conflicts of interest to report.

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●短 報

第54回日本呼吸器学会学術講演会 シンポジウム報告
IgG4関連呼吸器疾患の診断基準

松井 祥子[a] 山本 洋[b] 源 誠二郎[c]
早稲田優子[d] 三嶋 理晃[e] 久保 惠嗣[f]

要旨：IgG4関連呼吸器疾患の診断基準を，厚生労働省難治性疾患克服研究事業研究班より提案し，第54回日本呼吸器学会学術講演会シンポジウムにて討議した．診断項目は，画像所見・血液検査所見・病理所見・胸郭外臓器病変の存在，の4項目とし，診断を，確定診断群（definite)・準確診群（probable)・疑診群（possible）の3つに分類した．また解説とアルゴリズムを付記した．本診断基準の普及が望まれる．

キーワード：IgG4関連疾患，IgG4関連呼吸器疾患，診断基準
IgG4-related disease, IgG4-related respiratory disease, Diagnostic criteria

診断基準作成までの経緯

IgG4関連疾患は，高IgG4血症および病変部へのIgG4陽性形質細胞浸潤と線維化を特徴とする新しい全身性疾患である．2011年，厚生労働省難治性疾患克服研究事業研究班（厚労班）から，IgG4関連疾患包括診断基準が提唱され，現在では広く認知されている[1)2)]．

包括診断基準は，多臓器の共通所見をまとめた利便性の高い診断基準である．しかし医療が専門化・細分化されている現況から，厚労班では，各臓器病変の特異性に着目した「臓器別診断基準」の必要性も検討されており，すでに膵臓，腎臓などの臓器別診断基準が公表されている[3)4)]．

以上の経過から，厚労班呼吸器分科会では，呼吸器病変の診断基準作成を試みた．それを第54回日本呼吸器学会学術講演会（2014年4月，河野修興会長）のシンポジウムにおいて議論し，出席した会員との意見交換後，全員の同意を得て最終的な診断基準を作成したので，ここに報告する．

連絡先：松井 祥子
〒930-0194 富山県富山市杉谷2630
[a]富山大学保健管理センター杉谷支所
[b]信州大学学術研究院医学系医学部内科学第一教室
[c]大阪府立呼吸器アレルギー医療センターアレルギー内科
[d]金沢大学附属病院呼吸器内科
[e]京都大学大学院医学研究科呼吸器内科学
[f]地方独立行政法人長野県立病院機構
(E-mail: smatsui@med.u-toyama.ac.jp)
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IgG4関連呼吸器疾患の診療指針

1. 呼吸器病変の疾患名称

2011年にボストンで開催された国際シンポジウムにおいて，IgG4関連疾患の呼吸器病変は，「IgG4関連肺疾患（IgG4-related lung disease)」と「IgG4関連胸膜疾患(IgG4-related pleural disease)」という2つの個別の名称が採択された[5)]．しかしその後，呼吸器病変は広義間質病変であることが報告されたため[6)]，厚労班では，胸郭内の呼吸器および附属器の病変を包括して「IgG4関連呼吸器疾患（IgG4-related respiratory disease)」と呼称することとした．

2. IgG4関連呼吸器疾患の診断基準

呼吸器疾患の診断基準を表1に示した．

この診断基準の画像所見，臨床/検査所見，病理所見の把握のために，解説を付記した（表2)．

また，診断へ至る過程を具体的に示すためのアルゴリズムも作成した（図1)．

まとめ

IgG4関連呼吸器疾患の診断基準を報告した．今後は，本基準の普及が望まれる．

謝辞：本稿の作成にご協力いただきました京都大学 半田知宏先生に深謝します．本論文の研究は，厚生労働省難治性疾患克服研究事業の研究助成金によって行われた．

著者のCOI（conflicts of interest）開示：本論文発表内容に関して特に申告なし．

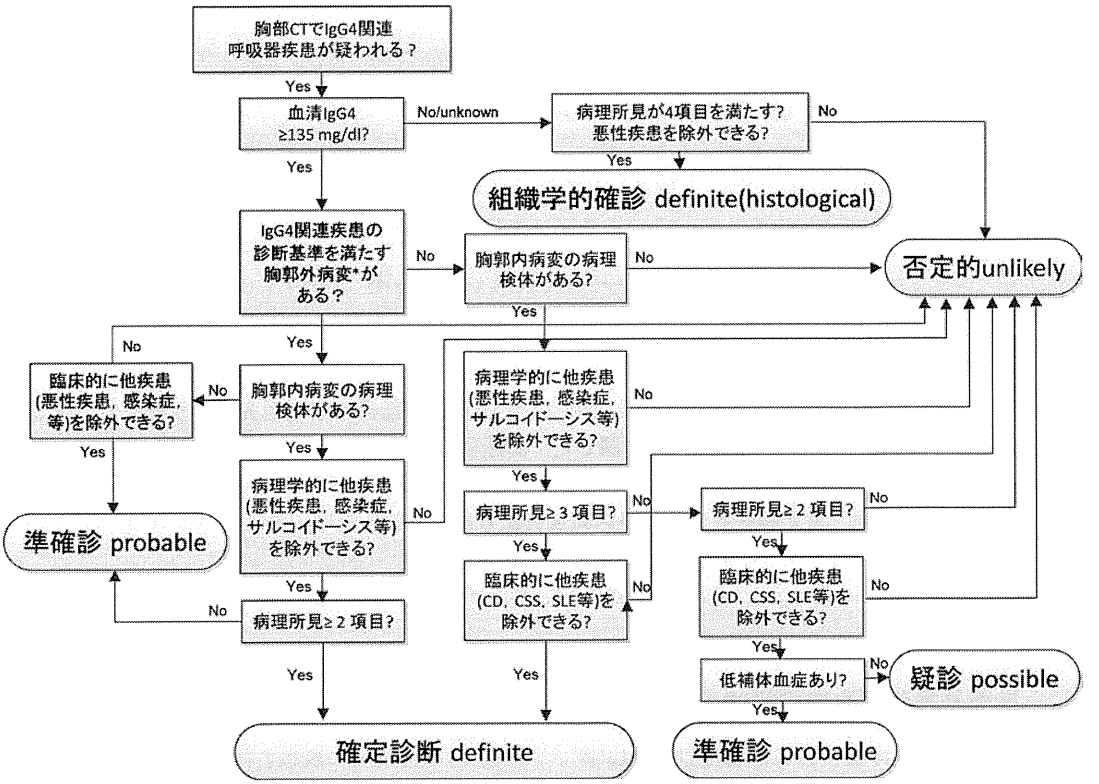

図1 診断のアルゴリズム．CD：Castleman disease（plasma cell type），CSS：Churg-Strauss syndrome，SLE：systemic lupus erythematosus．*硬化性涙腺炎・唾液腺炎，自己免疫性膵炎，IgG4 関連硬化性胆管炎，IgG4 関連腎臓病，後腹膜線維症．

表1 IgG4 関連呼吸器疾患診断基準

A．診断基準
1. 画像所見上，下記の所見のいずれかを含む胸郭内病変を認める
   肺門縦隔リンパ節腫大，気管支壁/気管支血管束の肥厚
   小葉間隔壁の肥厚，結節影，浸潤影，胸膜病変
2. 血清 IgG4 高値（135 mg/dl 以上）を認める
3. 病理所見上，呼吸器の組織において以下の①～④の所見を認める
   a：3 項目以上，b：2 項目
   ①気管支血管束周囲，小葉間隔壁，胸膜などの広義間質への著明なリンパ球，形質細胞の浸潤
   ②IgG4/IgG 陽性細胞比＞40%，かつ IgG4 陽性細胞＞10 cells/HPF
   ③閉塞性静脈炎，もしくは閉塞性動脈炎
   ④浸潤細胞周囲の特徴的な線維化*
4. 胸郭外臓器にて，IgG4 関連疾患の診断基準を満たす病変#がある

〈参考所見〉低補体血症

*自己免疫性膵炎診断基準の花筵状線維化に準ずる線維化所見

#硬化性涙腺炎・唾液腺炎，自己免疫性膵炎，IgG4 関連硬化性胆管炎，IgG4 関連腎臓病，後腹膜線維症

B．診　断
1. 確定診断（definite）：1＋2＋3a，1＋2＋3b＋4
   組織学的確定診断［definite（histological）］：1＋3-①～④すべて
2. 準確診（probable）：1＋2＋4，1＋2＋3b＋参考所見
3. 疑診（possible）：1＋2＋3b

C．鑑別診断
Castleman 病（plasma cell type），膠原病関連肺疾患，granulomatosis with polyangiitis（Wegener 肉芽腫症），eosinophilic granulomatosis with polyangitiis（Churg-Strauss 症候群），サルコイドーシス，呼吸器感染症，Rosai-Dorfman 病，inflammatory myofibroblastic tumor，悪性リンパ腫，肺癌　など

表2 付記：IgG4 関連呼吸器疾患診断基準の解説

1. 画像所見
・肺門・縦隔リンパ節腫大や気管支壁/気管支血管束の肥厚は頻度の高い所見である
・小葉間間質や胸膜を含む，いわゆる広義間質に病変を認める
・胸郭内の結節性，腫瘤性陰影や浸潤影として認められることがある
・画像所見は非特異的であるので，感染症や悪性疾患など鑑別診断に掲げた疾患を除外する必要がある
2. 臨床所見・検査所見
・アレルギー性鼻炎や気管支喘息などのアレルギー症状の既往や合併を伴うことがある
・高 IgG 血症，高 IgE 血症を伴うことが多いが，血清 IgA および IgM が同時に上昇することはまれである
・抗核抗体陽性，リウマチ因子陽性，低補体血症を認めることがある
・白血球増加や CRP 上昇などの炎症所見は認めないか，もしくは軽度異常にとどまる
3. 病理所見
・気管支血管束周囲の間質，小葉間隔壁，胸膜および連続する肺胞隔壁などの広義間質に，リンパ球，形質細胞の浸潤を伴う線維化巣を認める
・線維化の典型は「花筵状線維化」であり，リンパ球・形質細胞の浸潤を伴う紡錘形細胞の増生からなる．その方向性は無秩序で時に渦巻き状を呈する
・著明な細胞浸潤と線維化のため，肺胞腔を埋めるような腫瘤性病変が形成されることがある
・好酸球浸潤が散見されるが，好中球浸潤や肉芽腫は通常認めない
・病理診断には，外科的生検材料が望ましい
4. 胸郭外臓器病変
・胸郭外臓器病変は，確立された臓器別診断基準を満たす病変（膵臓，胆管，腎臓），あるいは病理所見にて著明なリンパ球・IgG4 陽性形質細胞浸潤と線維化を伴い特徴的な臨床・画像所見を示す病変（涙腺・唾液腺，後腹膜）である

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Abstract

Diagnostic criteria for IgG4-related respiratory disease

Shoko Matsui[a], Hiroshi Yamamoto[b], Seijiro Minamoto[c], Yuko Waseda[d], Michiaki Mishima[e] and Keishi Kubo[f]

[a]University of Toyama
[b]Shinshu University School of Medicine
[c]Osaka Prefectural Medical Center for Respiratory and Allergic Diseases
[d]Kanazawa University Graduate School of Medical Science
[e]Graduate School of Medicine, Kyoto University
[f]Nagano Prefectural Hospital Organization

The diagnostic criteria for IgG4-related respiratory disease were proposed by the Subcommittee of Respiratory Disease of IgG4-related Disease supported by the Health and Labor Sciences Research Grants for the Study of Intractable Diseases from the Ministry of Health, Labour and Welfare, Japan. The criteria include the following 4 conditions: 1) image findings, 2) blood test findings, 3) pathological findings, and 4) presence of extra-thoracic organ lesions, and are classified in three stages of definite, probable, and possible according to combinations of the above conditions. Also, we added a commentary and algorithm to the diagnostic criteria. The criteria were presented in the symposium of the 54th Annual Meeting of the Japanese Respiratory Society (2014) and discussed by members of the respiratory society. The diagnostic criteria and algorithm will be useful for clarifying the entity of IgG4-related respiratory disease.

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The Japanese Society of Gastroenterology

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Comparison study of immunohistochemical staining for the diagnosis of type 1 autoimmune pancreatitis

Katsuyuki Miyabe · Kenji Notohara · Takahiro Nakazawa · Kazuki Hayashi · Itaru Naitoh · Shuya Shimizu · Hiromu Kondo · Michihiro Yoshida · Hiroaki Yamashita · Shuichiro Umemura · Yasuki Hori · Akihisa Kato · Satoru Takahashi · Hirotaka Ohara · Takashi Joh

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Abstract
Background Various methods to evaluate immunohistochemical staining (IHC) for the diagnosis of type 1 autoimmune pancreatitis (AIP) have been proposed. Our goal was to determine the most useful IHC method for the diagnosis of AIP.
Methods Specimens of AIP (18 patients), chronic pancreatitis (CP, 24 patients), and pancreatic ductal adenocarcinoma (PDA, 45 patients) were evaluated with IHC for immunoglobulin G (IgG), IgG1, IgG4, and CD138 (syndecan-1). The number of IHC-positive cells was counted in 3, 5, and 10 different high-power fields (HPFs) by selecting fields with the most numerous positive cells (hotspot) or by randomly selecting fields in the affected areas (random). We evaluated the mean number of IgG4-positive plasma cells (IgG4+)/HPF (mean IgG4+), the number of fields with >10 and >50 IgG4+ (NOF >10 and NOF >50 IgG4+), the ratio of IgG4+/IgG+, IgG4+/IgG1+, and IgG4+/CD138+.
Results Analysis with receiver operator characteristic curves revealed that accurate and practical parameters in 3 HPFs were mean IgG4+ with the hotspot method (sensitivity, 88.9; specificity, 92.8 %), mean IgG4+ with the random method (100, 95.7 %), and NOF >10 IgG4+ with the random method (94.4, 97.1 %). These results were as accurate as results from 5 HPFs to 10 HPFs. The combination of mean IgG4+ and IgG4+/IgG+ did not provide more accurate diagnosis for AIP than a single criterion itself.
Conclusions Mean IgG4+ or NOF >10 IgG4+ with the random method in 3 HPFs was a useful and simple diagnostic method for AIP. The combined criteria of mean IgG4+ and IgG4+/IgG+ might not be required for accurate diagnosis of AIP.

Keywords Autoimmune pancreatitis · IgG4-related disease · Lymphoplasmacytic sclerosing pancreatitis · Immunohistochemical staining

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K. Miyabe · T. Nakazawa (✉) · K. Hayashi · I. Naitoh · S. Shimizu · H. Kondo · M. Yoshida · H. Yamashita · S. Umemura · Y. Hori · A. Kato · T. Joh
Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
e-mail: tnakazaw@med.nagoya-cu.ac.jp

K. Miyabe
e-mail: kmiyabe@med.nagoya-cu.ac.jp

K. Notohara
Department of Pathology, Kurashiki Central Hospital, Kurashiki, Japan

S. Takahashi
Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

H. Ohara
Department of Community-based Medical Education, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Introduction

Autoimmune pancreatitis (AIP) has been classified as type 1 (lymphoplasmacytic sclerosing pancreatitis, LPSP) or type 2 AIP (idiopathic duct-centric pancreatitis, IDCP, or AIP with granulocytic epithelial lesion, GEL). Numerous IgG4-

positive (IgG4+) plasma cells in affected tissues are characteristic of type 1 AIP, and an increased number of IgG4+ plasma cells is used as a diagnostic standard for type 1 AIP by many pathologists. This finding, however, is not entirely specific for type 1 AIP because some cases of other pancreatic diseases such as pancreatic cancer or non-specific chronic pancreatitis have affluent IgG4+ plasma cell infiltrations [1–4].

Since a large number of studies about immunohistochemical staining (IHC) in IgG4-related disease (IgG4-RD) have been published, the degree of IgG4+ cell infiltration has been incorporated into various diagnostic criteria. Some diagnostic criteria of type 1 AIP adopted the findings >10 IgG4+ plasma cells/high power field (HPF) as one of the pathological criteria of type 1 AIP [5–7], while other criteria of IgG4-RD mentions that the appropriate cut-off point may vary from organ to organ and the findings of >50 IgG4+ plasma cells/HPF are highly specific in resected pancreas specimens [8]. Thus, the most appropriate number of field counts has been controversial.

The ratio of IgG4+/IgG-positive plasma cells (IgG4+/IgG+) >40 % has been adopted as the histological diagnostic criteria for IgG4-RD [8, 9], although the ratio of IgG4+/IgG+ was inconsistent in some papers. Other papers also made statements about the usefulness of the ratio of IgG4+/IgG1-positive plasma cells (IgG4+/IgG1+) in differentiating IgG4-sclerosing cholangitis from primary sclerosing cholangitis [10] and the ratio of IgG4+/CD138-positive plasma cells (IgG4+/CD138+) in the gastrointestinal mucosa for the diagnosis of IgG4-RD [11].

Furthermore, it has been speculate that hotspot counting might be the best because random counting might result in underestimation of IgG4+ cells if there are many intervening microscopic fields without IgG4+ cells [8]; however, no previous reports have directly compared these two methods. In the present study, we evaluated methods of IHC to determine which method is the most useful for the diagnosis of type 1 AIP.

Materials and methods

Patients

The files of the Department of Pathology, Nagoya City University Graduate School of Medicine, Kurashiki Central Hospital, and affiliated hospitals were searched for pancreas resections for which the final diagnosis was chronic pancreatitis. All cases underwent an operation with a clinical diagnosis of pancreatic cancer. Excisional biopsy specimens larger than 1 cm^2 obtained by surgery were also included in this study, because these materials contained sufficient pancreatic and stromal tissue for infiltrated inflammatory cells to be counted.

As previously reported [12], we gathered 18 cases of type 1 AIP, 24 cases of chronic pancreatitis (CP), and 45 cases of pancreatic adenocarcinoma (PDA). All hematoxylin and eosin (HE)-stained slides were reviewed by two authors (KM and KN) without clinical information, and cases of type 1 AIP and CP were identified. All of the type 1 AIP cases were focal involvement of the pancreas showing the full-blown histologic features: abundant (>10 cells/HPF) IgG4+ plasma cells, periductal lymphoplasmacytic infiltration, storiform fibrosis and obliterative phlebitis on resected specimens. The first of these will be discussed in detail in this manuscript. The CP cases consisted of both alcoholic and idiopathic CP and were diagnosed on the basis of the Zurich classification [13] such as a history of excessive alcohol intake, calcification in the pancreas, or moderate to marked ductal lesions described in the Cambridge classification [14]. Cases without an apparent cause for chronic pancreatitis were classified as idiopathic. Pathology of chronic pancreatitis was characterized by perilobular fibrosis and acinar destruction with acute and chronic inflammatory cells. Resected specimens of pancreas adenocarcinoma were also included in the present study.

This study was approved by the review board of Nagoya City University (approval No. 284-2).

Histology and IHC

The resected specimens were fixed in formalin and embedded in paraffin. A representative tissue block with the most remarkable fibrosis and inflammatory cells infiltration was selected from all slides of each case. Serial 3-μm thick sections were made from paraffin to embedded tissue blocks, stained with hematoxylin and eosin, and immunostained with rabbit anti-human IgG polyclonal antibody (dilution 1:10000; Dako, Glostrup, Denmark), sheep anti-human IgG1 monoclonal antibody (AU006, dilution 1:100; The Binding Site Limited, Birmingham, UK), or mouse anti-human IgG4 monoclonal antibody (MC011, dilution 1:100; The Binding Site Limited, Birmingham, UK), mouse anti-human CD138 monoclonal antibody (MI15, dilution 1:200; Dako Cytomation, Glostrup, Denmark) by using the DISCOVRY HX (Ventana Medical Systems, Tuscon, AX, USA), and Bond Max (Leica Microsystems, Wetzlar, Germany) automated immunostainers.

All slides were reviewed in a blinded manner by two independent authors (KM and HY) without clinical information. When the assessment was different between the two observers, agreement was reached by using a double-headed microscope. These slides were observed by light microscopy (Nikon ECLIPSE 80i, Nikon Corporation Tokyo, Japan) with a 40× field objective and 10× ocular

lens, which correspond to a field diameter of 550 μm for the slides.

Counting methods for IgG4+ plasma cells

We determined the mean number of IgG4+ plasma cells/HPF (mean IgG4+) in each case and the number of fields with more than 10 and 50 IgG4+ plasma cells/HPF (NOF >10 and 50 IgG4+) by using the hotspot and random method, as shown in Fig. 1. For the hotspot method (Fig. 1b), IgG4+ plasma cells were counted in 10 different HPFs by selecting fields with the most numerous IgG4+ plasma cells. For the random method (Fig. 1c), the number of IgG4+ plasma cells was counted in 10 different HPFs by randomly selecting fields with intense inflammation.

IHC-positive cell ratio

We also measured the ratio (%) parameters of IgG4+/IgG+, IgG4+/IgG1+, and IgG4+/CD138+ with the hotspot and random methods. To evaluate the ratios of IHC-positive cells in the same fields, 10 different HPFs of IgG4+ plasma cells with 100 μm scale were scanned by using a BIOREVO BZ-9000 microscope (KEYENCE Corp., Osaka, Japan) with each counting method, and the number of these cells per 100 μm^2 was counted by using the rectangle tool of Adobe® Photoshop CS5 extended software (version 11.0.2; Adobe Systems, Berkeley, CA). IgG, IgG1, and CD138-positive plasma cells were also scanned in the same 10 HPFs as those of IgG4+ plasma cells and the numbers of these cells were counted in the same way (Fig. 2).

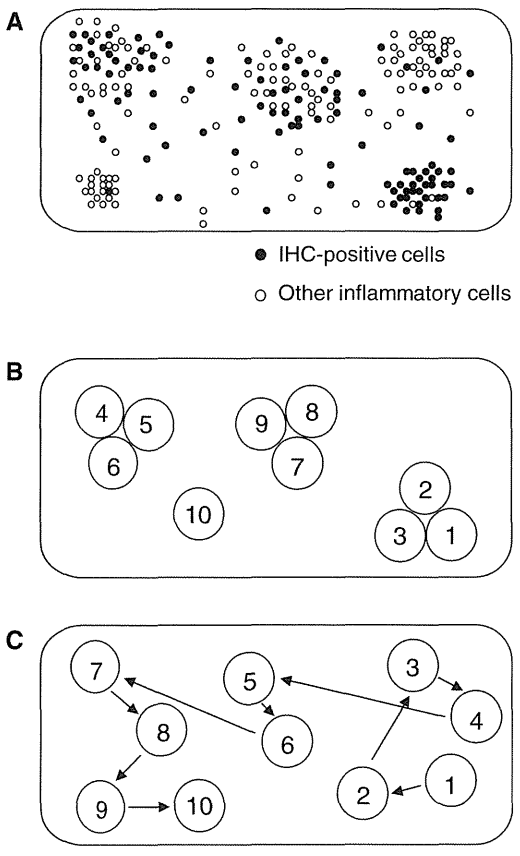


Fig. 1 Illustration of the way to count IHC-positive cells. **a** Schematic distribution of IgG4+ plasma cells and other inflammatory cells. The number of IHC-positive cells was counted in 10 HPFs by **b** selecting fields with the most numerous positive cells (hotspot), or by **c** randomly selecting fields in the affected areas (random)

The number of fields counted

We further assessed whether the number of fields counted influenced the accuracy of the diagnosis of type 1 AIP. The parameters mentioned above were calculated according to 3, 5, and 10 HPFs and then compared. The significance of each parameter between the hotspot and random methods, and between 3, 5, and 10 HPFs were compared, and the best cut-off values and area under curves (AUCs) to differentiate type 1 AIP from CP to PDA were evaluated by the receiver operator characteristic (ROC) curves.

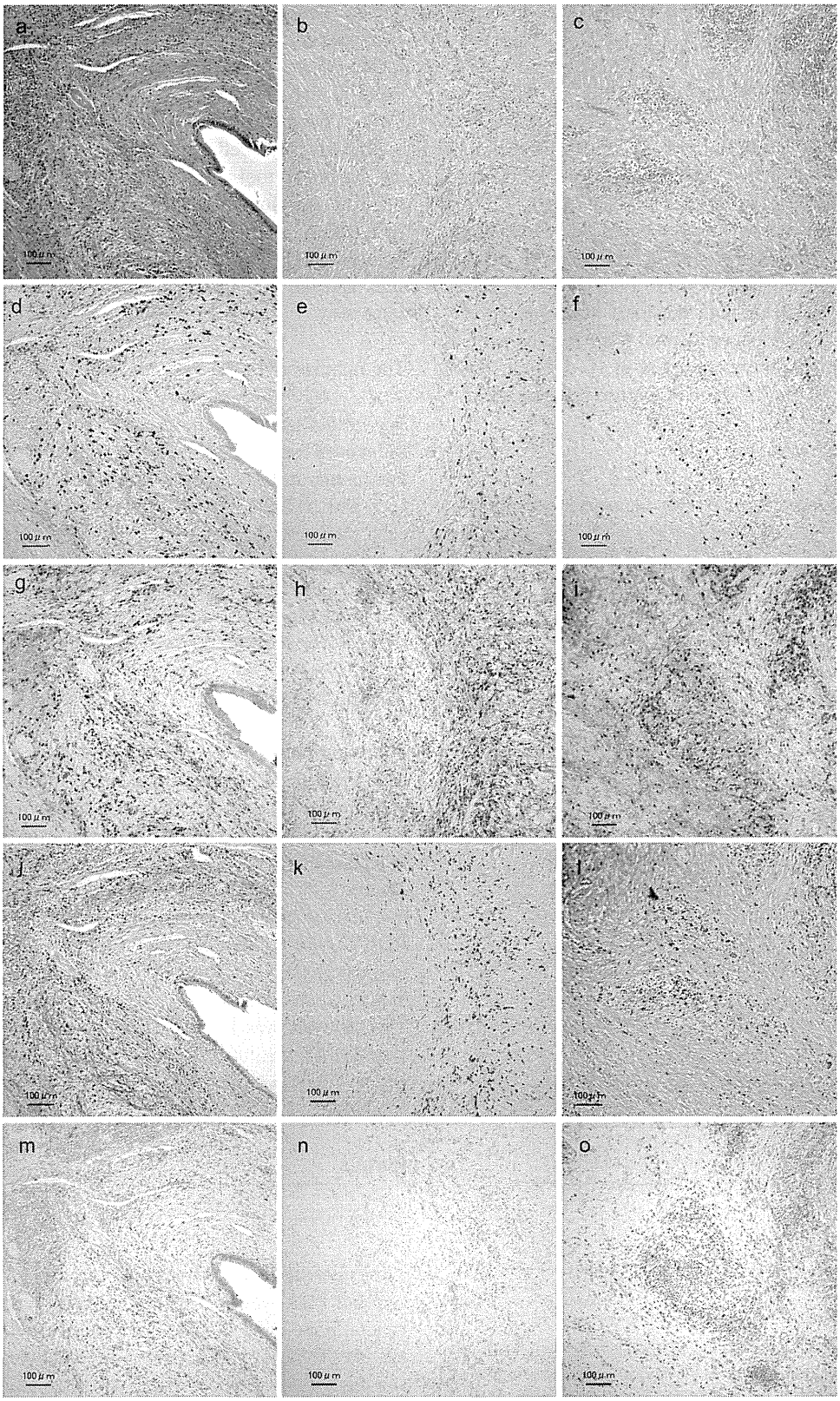
Statistical analysis

Statistical analysis was performed with non-parametric tests because of the non-Gaussian distribution of the data and smaller sample sizes in some comparisons. Dunnett's test was used for comparisons between type 1 AIP and CP/PDA and the Tukey–Kramer test was used for comparisons among 3, 5, and 10 HPFs, if the Kruskal–Wallis test for comparisons of three groups showed significant results. For ROC curves, the best cut-off values were chosen according to the highest diagnostic accuracy determined by using the Youden index with sensitivity − (1 − specificity) [15, 16]. This analysis was performed with JMP software (version 10.0.2, SAS Institute, Cary, NC). All tests were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Demographics and clinical presentation

Clinical characteristics for the present series of type 1 AIP, CP, and PDA were not greatly different from those in the general population as mentioned in the previous paper [12, 17–23] (Table 1). CP patients were younger ($P < 0.0001$) and PDA patients more likely to be female as compared with other groups (type 1 AIP vs PDA, $p = 0.0258$; CP vs PDA, $p = 0.0065$).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◀**Fig. 2** Representative figures with numerous IgG4-positive plasma cells in type 1 AIP (**a**, **d**, **g**, **j**, **m**), CP (**b**, **e**, **h**, **k**, **n**), and PDA (**c**, **f**, **i**, **l**, **o**) on HE (**a**, **b**, **c**) and the IHCs of IgG4 (**d**, **e**, **f**), IgG (**g**, **h**, **i**), IgG1 (**j**, **k**, **l**), and CD138 (**m**, **n**, **o**). Original magnification ×100

Table 1 Clinical data of the cases used in this study

	Type 1 AIP ($n = 18$)	CP ($n = 24$)	PDA ($n = 45$)
Age (mean, range)	66 (54–79)	48.5 (14–67)*	67 (32–82)
Sex (male/female)	17/1	23/1	30/15†
Sign and symptoms			
Obstructive jaundice	10	0	15
Abdominal pain or back pain	6	19	17
Excessive alcohol intake	2 (NA, 3 cases)	18	3 (NA, 1 case)
Radiographic findings			
Mass forming	18	7	45
MPD stricture	15 (NA, 3 cases)	18 (NA, 2 cases)	37
MPD dilatation	4 (NA, 3 cases)	20 (NA, 2 cases)	37
CBD stricture	13	4	25
Calcification in the pancreas	0	16	1
Surgical procedure			
PD or PpPD	13	7	27
DP	4	16	17
Excisional biopsy	1	1	1

MPD main pancreatic duct, *CBD* common biliary duct, *PD* pancreaticoduodenectomy, *PpPD* pylorus-preserving pancreaticoduodenectomy, *DP* distal pancreatectomy and splenectomy, *NA* not available. Patients who had consumed >80 g/day of alcohol for some years were considered to have excessive alcohol intake

* $p < 0.0001$ as compared with other groups, † $p = 0.0258$ vs type 1 AIP and $p = 0.0065$ vs PDA

Comparison of hotspot and random counting among the disease groups

We counted IgG4+, IgG1+, IgG+, and CD138+ plasma cells in 3, 5, and 10 hotspot and random HPFs (Supplementary table S1). Mean IgG4+, NOF >10 IgG4+, and NOF >50 IgG4+ with both counting methods were significantly greater in type 1 AIP than in CP and PDA (all comparisons, $p < 0.0001$). With hotspot counting, IgG4+/IgG+ and IgG4+/IgG1+ were significantly higher in type 1 AIP than in CP and PDA ($p < 0.05$), whereas IgG4+/CD138+ did not differ significantly among the three disease groups. With the random counting, IgG4+/IgG+, IgG4+/IgG1+, and IgG4+/CD138+ were significantly higher in type 1 AIP than in CP and PDA (all comparisons, $p < 0.0001$).

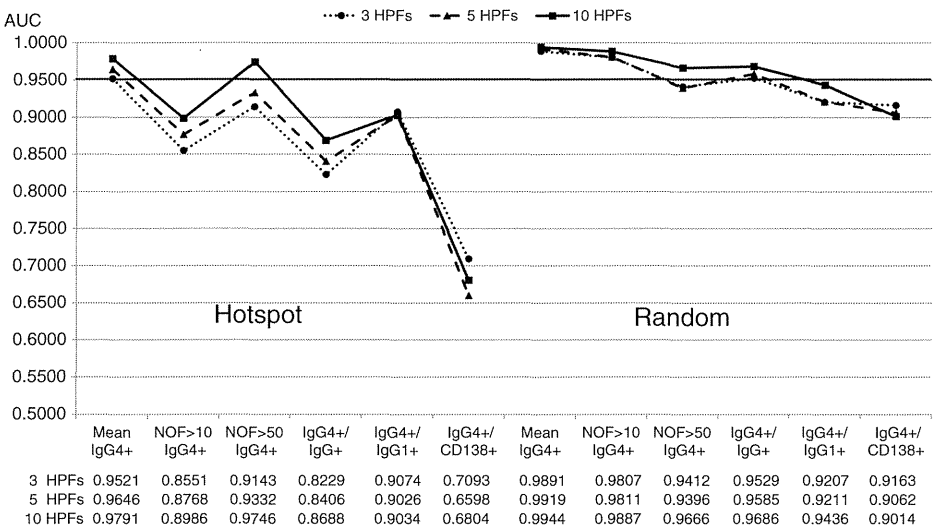
Group comparisons among the numbers of fields counted (3, 5 and 10 HPFs)

In terms of mean IgG4+ and the ratio of IHC-positive cells, no significant difference among the numbers of fields counted was detected in all disease groups with both counting methods (Supplementary table S1). Among NOF >10 IgG4+ with both counting methods in type 1 AIP, and NOF >50 IgG4+ with the hotspot method in type 1 AIP, the significant largest number was observed in 10 HPFs, followed by in 5 HPFs and in 3 HPFs (all comparisons, $p < 0.0001$), whereas no significant difference among the numbers of fields counted was detected in CP and PDA with either counting method. In type 1 AIP with the random method, NOF >50 IgG4+ in 10 HPFs was significantly larger than in 5 HPFs and in 3 HPFs (both, $p < 0.01$), whereas there was no significant difference between 3 HPFs and 5 HPFs.

Comparison of AUC and diagnostic accuracy

We performed ROC analysis for each parameter (Fig. 3). Among the numbers of fields counted, the largest AUC for each parameter was obtained for 10 HPFs, except IgG4+/IgG1+ with the hotspot method, NOF >50 IgG4+ with the random method and IgG4+/CD138+ with both counting methods, followed by 5 HPFs and 3 HPFs. However, most of the parameters, especially mean IgG4+ with both counting methods, IgG4+/IgG1+ with the hotspot method, and NOF >10 IgG4+ and IgG4+/IgG+ with the random method showed subtle AUC differences among those in 3, 5, and 10 HPFs. The largest AUC was obtained for mean IgG4+ with the random method. Mean IgG4+ with both counting methods, NOF >10 IgG4+ with the random method, and IgG4+/IgG+ with the random method had as AUC >0.9500 in 3, 5, and 10 HPFs. In NOF >50 IgG4+ with both counting methods, only those in 10 HPFs showed AUCs >0.9500. These parameters were indicated very precise for the diagnosis of type 1 AIP. IgG4+/CD138+ was inferior to any other parameter with either counting method.

Optimal cut-off values for all parameters in 3 HPFs for differentiating type 1 AIP from CP/PDA are shown in Table 2. These cut-off values (sensitivity/specificity and AUC) of parameters with AUC >0.9500 were: 68.3 (88.9/92.8 %, 0.9521) for mean IgG4+ with the hotspot method, 9.667 (100/95.7 %, 0.9891) for mean IgG4+ with the random method, and 2 fields (94.4/97.1 %, 0.9807) for NOF >10 IgG4+ with the random method, 18.421 % (94.4/88.4 %, 0.9529) for IgG4+/IgG+ with the random method. Mean IgG4+ >10 was exhibited for 18 cases of type 1 AIP, 8 cases of CP, and 24 cases of PDA with the hotspot method (sensitivity 100 % and specificity 53.6 %



	Mean IgG4+	NOF>10 IgG4+	NOF>50 IgG4+	IgG4+/ IgG+	IgG4+/ IgG1+	IgG4+/ CD138+	Mean IgG4+	NOF>10 IgG4+	NOF>50 IgG4+	IgG4+/ IgG+	IgG4+/ IgG1+	IgG4+/ CD138+
3 HPFs	0.9521	0.8551	0.9143	0.8229	0.9074	0.7093	0.9891	0.9807	0.9412	0.9529	0.9207	0.9163
5 HPFs	0.9646	0.8768	0.9332	0.8406	0.9026	0.6598	0.9919	0.9811	0.9396	0.9585	0.9211	0.9062
10 HPFs	0.9791	0.8986	0.9746	0.8688	0.9034	0.6804	0.9944	0.9887	0.9666	0.9686	0.9436	0.9014

Fig. 3 AUC value of each parameter. The *numbers* under the *horizontal axis* indicate AUC values

for the diagnosis of type 1 AIP) and 17 cases of type 1 AIP, 0 cases of CP, and 2 cases of PDA cases with the random method (94.4 and 95.7 %, respectively). Mean IgG4+ >50 was exhibited for 16 cases of type 1 AIP, 2 cases of CP, and 4 cases of PDA with the hotspot method (88.9 and 91.3 %, respectively) and 9 cases of type 1 AIP, 0 cases of CP, and 0 cases of PDA with the random method (50.0 and 100 %, respectively).

We also performed ROC analysis using only type 1 AIP and PDA groups in order to investigate the usefulness in clinical practice. Only mean IgG4+ and NOF >10 IgG4+ with the random method had AUC >0.9500 in 3, 5, and 10 HPFs and these cut-off values (sensitivity/specificity and AUC) in 3 HPFs were: 9.667 (100/93.3 %, 0.9833) for mean IgG4+ with the random method, and 2 fields (94.4/95.6 %, 0.9701) for NOF >10 IgG4+ with the random method.

We further studied whether the combination of mean IgG4+ and IgG4+/IgG+ in 3 HPFs could lead to improved diagnostic accuracy for type 1 AIP as compared with each single criterion (Fig. 4). Mean IgG4+ >10 and IgG4+/IgG+ >40 % were exhibited by 15 cases of type 1 AIP, 2 cases of CP, and 9 cases of PDA with the hotspot method (sensitivity 83.3 %, specificity 84.1 %), while mean IgG4+ >50 and IgG4+/IgG+ >40 % were exhibited by 14 cases of type 1 AIP, 1 case of CP, and 2 cases of PDA with the hotspot method (sensitivity 77.8 %, specificity 95.7 %). When the best cut-off values were applied for these criteria, 8 cases of type 1 AIP, 1 case of CP, and 0 cases of PDA showed mean IgG4+ >68.3 and IgG4+/IgG+ >58.3 % with the hotspot method (sensitivity 44.4 %, specificity 98.6 %), and 16 cases of type 1 AIP, 0 cases of CP, and 0 cases of PDA showed mean IgG4+ >9.7 and IgG4+/IgG+ >18.4 % with the random method (sensitivity 88.9 %, specificity 100 %). These results indicate that combined criteria provide higher specificity and lower sensitivity than each single criterion but were unable to provide a more accurate diagnosis for AIP than some of the single criterions, such as mean IgG4+ and NOF >10 IgG4+ with the random method.

Discussion

We investigated the number of IgG4+ plasma cells and the ratio of IgG4+/IgG+, IgG4+/IgG1+, and IgG4+/CD138+ on slides selected with the hotspot and random methods, and we clarified that the random counting was not inferior to the hotspot counting. In previous papers, IgG4+ cells >10 [3, 6, 24–28], >20 [29], >30 [30, 31], >50 [1, 32, 33], and >100 per HPF [34] and the ratio of IgG4+/IgG+ cells >10 % [35], >40 % [26, 30, 36], and >50 % [37] have been proposed as criteria for a diagnosis of IgG4-RD (Table 3). This present study revealed that all parameters except IgG4+/CD138+ with the hotspot method were significantly greater in type 1 AIP than in CP and PDA with both counting methods. These results indicate that all ratios except IgG4+/CD138+ with the hotspot method may be useful and practical diagnostic markers for type 1 AIP. This finding reflects the observation that tissues from patients with IgG4-RD show diffuse infiltrates of IgG4+ plasma cells, in contrast to the focal aggregates of IgG4+ plasma cells that are detected in most inflammatory mimickers of this condition [38].

Table 2 Outcome analyzed receiver-operating characteristic curves for each parameter

	Cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC (95 % CI)
(a) Hotspot					
Mean IgG4+					
3 HPFs	68.300	88.9	92.8	90.8	0.9521 (0.8885–0.9802)
5 HPFs	53.400	94.4	89.9	87.7	0.9646 (0.9042–0.9874)
10 HPFs	54.600	94.4	92.8	93.6	0.9791 (0.9331–0.9937)
Current criteria 1					
3 HPFs	10.000	100	53.6	76.8	
5 HPFs	10.000	100	55.1	77.5	
10 HPFs	10.000	100	55.2	77.6	
Current criteria 2					
3 HPFs	50.000	88.9	91.3	90.1	
5 HPFs	50.000	94.4	88.4	95.3	
10 HPFs	50.000	94.4	91.3	92.9	
NOF >10 IgG4+ (fields)					
3 HPFs	3	100	71.0	85.5	0.8551 (0.7925–0.9011)
5 HPFs	5	100	75.4	87.7	0.8768 (0.8159–0.9196)
10 HPFs	10	100	79.7	89.9	0.8986 (0.8398–0.9374)
NOF >50 IgG4+ (fields)					
3 HPFs	2	88.9	91.3	90.1	0.9143 (0.8105–0.9637)
5 HPFs	3	94.4	91.3	92.9	0.9332 (0.8170–0.9776)
10 HPFs	6	94.4	91.3	92.9	0.9746 (0.9216–0.9921)
IgG4+/IgG+ (%)					
3 HPFs	58.333	88.9	72.5	80.7	0.8229 (0.7111–0.8976)
5 HPFs	52.147	83.3	76.8	80.1	0.8406 (0.7291–0.9117)
10 HPFs	36.957	94.4	73.9	84.2	0.8688 (0.7545–0.9345)
IgG4+/IgG1+ (%)					
3 HPFs	90.667	88.9	85.5	87.2	0.9074 (0.8054–0.9587)
5 HPFs	88.136	88.9	82.6	85.8	0.9026 (0.8077–0.9534)
10 HPFs	78.889	94.4	79.7	87.1	0.9034 (0.8139–0.9524)
IgG4+/CD138+ (%)					
3 HPFs	46.281	83.3	60.9	72.1	0.7093 (0.5833–0.8097)
5 HPFs	53.125	83.3	55.1	69.2	0.6598 (0.5264–0.7720)
10 HPFs	43.463	88.9	55.1	72.0	0.6804 (0.5448–0.7910)
(b) Random					
Mean IgG4+					
3 HPFs	9.667	100	95.7	97.8	0.9891 (0.9548–0.9975)
5 HPFs	11.200	100	97.1	98.6	0.9919 (0.9618–0.9983)
10 HPFs	15.667	100	97.1	98.6	0.9944 (0.9692–0.9990)
Proposed cut-off value					
3 HPFs	10.000	94.4	95.7	95.1	
5 HPFs	10.000	100	97.1	98.6	
10 HPFs	10.000	100	97.1	98.6	
NOF >10 IgG4+ (fields)					
3 HPFs	2	94.4	97.1	95.8	0.9807 (0.9317–0.9947)
5 HPFs	2	100	95.7	97.8	0.9811 (0.9285–0.9952)
10 HPFs	5	100	97.1	98.6	0.9887 (0.9358–0.9981)
NOF >50 IgG4+ (fields)					
3 HPFs	1	88.9	98.6	93.7	0.9412 (0.8031–0.9534)

Table 2 continued

	Cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC (95 % CI)
5 HPFs	1	88.9	97.1	93.0	0.9396 (0.8012–0.9836)
10 HPFs	1	94.4	97.1	95.8	0.9666 (0.8351–0.9940)
IgG4+/IgG+ (%)					
3 HPFs	18.421	94.4	88.4	91.4	0.9529 (0.8647–0.9846)
5 HPFs	16.854	94.4	88.4	91.4	0.9585 (0.8984–0.9837)
10 HPFs	20.7	94.4	94.2	94.3	0.9686 (0.9153–0.9888)
IgG4+/IgG1+ (%)					
3 HPFs	23.333	94.4	82.6	88.5	0.9207 (0.8388–0.9628)
5 HPFs	19.565	100	76.8	88.4	0.9211 (0.8449–0.9616)
10 HPFs	28.6	94.4	84.1	89.3	0.9436 (0.8634–0.9776)
IgG4+/CD138+ (%)					
3 HPFs	17.949	94.4	76.8	85.6	0.9163 (0.8225–0.9628)
5 HPFs	16.279	100	72.5	86.2	0.9062 (0.8223–0.9528)
10 HPFs	23.7	88.9	82.6	85.8	0.9014 (0.8147–0.9500)

Current criteria 1 in the hotspot method is based on the International Consensus Diagnostic Criteria, the HISORt Criteria, the Japanese Diagnostic Criteria, and the Asian Criteria. Current criteria 2 in the hotspot method is based on the Consensus statement on the pathology of IgG4-related disease. Proposed cut-off values in the random method are based on the results of the present study

95 % CI 95 % confidence interval

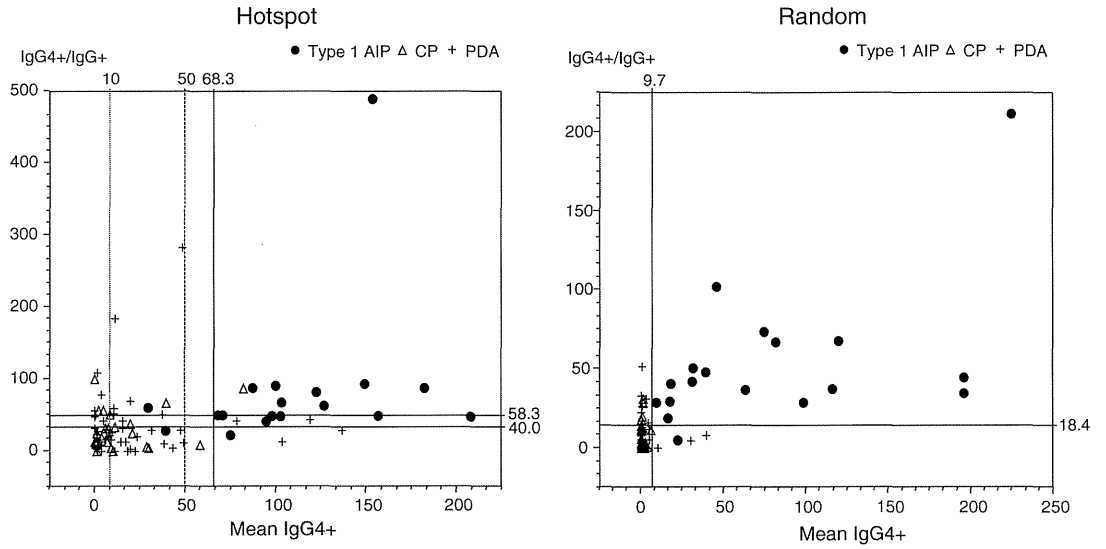


Fig. 4 Scatter plot of relationship between mean IgG4+ and IgG4+/IgG+ with the hotspot and random counts (3 HPFs). The cut-off values are based on the diagnostic criteria for IgG4-RD (the hotspot method, mean IgG4+ >10 or 50, IgG4+/IgG+ >40) and the present study's outcome (the hotspot method, mean IgG4+ >68.3 and IgG4+/IgG+ >58.3; the random method, mean IgG4+ >9.7 and IgG4+/IgG+ >18.4)

Additionally, we elucidated that 3 HPFs for the count fields yielded almost equal accuracy with 5 HPFs and 10 HPFs. The most appropriate number of count fields has not been elucidated so far, although the IHC-positive cells in at least 1 HPF [39], 3 HPFs [3, 29, 32, 33, 40], 5 HPFs [11, 41, 42], and 10 HPFs [4, 24, 35, 37] have been evaluated (Table 3). In our comparison among the numbers of counted fields, no significant differences among 3, 5, and 10 HPFs were detected in mean IgG4+, IgG4+/IgG+, IgG4+/IgG1+, and IgG4+/CD138+ for either counting method. Significant differences among 3, 5, and 10 HPFs of NOF >10 and 50 IgG4+ plasma cells in type 1 AIP were merely due to the different number of counted fields among them.

Table 3 Previous papers on the number of IgG4+ plasma cells and IgG4+/IgG+ ratio for the diagnosis of LPSP

References	IgG4+ plasma cell counts	Method for counting IgG4+ plasma cells	Investigated organs	IgG4+/IgG+	Sampling procedure
Deshpande et al. [1]	>50 per 20×	The area with the highest IgG4 score	Pancreas	NA	Resection
Zhang et al. [3]	>10/HPF	3 HPFs	Pancreas	NA	Resection
Bang et al. [4]	Non-specific	10 HPFs	Pancreas	NA	Resection or biopsy
Rebours et al. [11]	Non-specific	5HPFs at least	Stomach, duodenum, Ileum, and colon	NA	Biopsy
Deheragoda et al. [24]	>10/HPF	10 HPFs (at least 5 HPF), densest LPSP infiltrate	Pancreas and 7 other organs	NA	Resection or biopsy
Detlefsen et al. [25]	>10/HPF	1 HPF at least	Pancreas	NA	Biopsy
Kawano et al. [26]	>10/HPF	5 HPFs with intensive infiltration	Kidney	>40 %	Biopsy
Iwashita et al. [27]	>10/HPF	1 HPF	Pancreas	NA	Biopsy
Kanno et al. [28]	>10/HPF	10 HPFs selected randomly or all HPFs available	Pancreas	NA	Biopsy
Kojima et al. [29]	>20/HPF	3 HPFs in periductal areas	Pancreas	NA	Resection
Stone et al. [30]	>30/HPF	Not described	Described as systemic disease	>40 %	Resection or biopsy
Kamisawa et al. [31]	>30/HPF	Not described	Pancreas and 5 other organs	NA	Biopsy
Dhall et al. [32]	>50/HPF	3 HPFs in the highest density area	Pancreas	NA	Resection
Zen et al. [33]	>50/HPF	3 HPFs with intense inflammation	Pancreas and other 15 organs	>30 %	Resection
Yamamoto et al. [34]	>100/HPF (40×)	3 HPFs in the highest density area	Pancreas and other 4 organs	>50 %	Resection or biopsy
Sepehr et al. [35]	Not described	6 HPFs (the most superficial and central areas) and 4 HPF (adjacent and deeper areas)	Vater ampulla	>0.10 (10 %)	Resection
Cheuk et al. [36]	Not described	3 HPFs in the highest density area	Lymph node	>40 %	Resection
Zen et al. [37]	Not described	10 HPFs with intense inflammation	Lung	NA	Resection or biopsy
Chen et al. [40]	Not described	3HPFs at least in the highest density area	Mesentery	NA	Resection or biopsy
Kitagawa et al. [41]	Not described	5 HPFs with intense inflammation	Salivary gland	>45 %	Resection or biopsy
Strehl et al. [42]	Non-specific	5 HPFs with the highest plasma cell infiltration	Salivary gland	Non-specific	Resection

NA not available

A comparison of AUC for the diagnosis of type 1 AIP also indicated that the AUCs among 3, 5, and 10 HPFs were close on all parameters. Particularly, mean IgG4+ with the hotspot and random methods, NOF >10 IgG4+ with the random method, and IgG4+/IgG+ with the random method had excellent accuracy (AUC >0.9500) even in 3 HPFs although the value in 10 HPFs was the most accurate among them. In these parameters, IgG4+/IgG+ with the random method is not practical for clinical use, albeit they are highly specific, because it is difficult to count and calculate, especially in a clinical setting. One purpose of the present study was to determine if IgG4+/IgG1+ or IgG4+/CD138+ might be a new useful criterion for the diagnosis of type 1 AIP. However, IgG4+/IgG1+ was not able to surpass the accuracy of IgG4+/IgG+, and IgG4+/CD138+ was inferior to all other parameters with each method.

Particularly, we would like to emphasize that the cut-off value for mean IgG4+ with the random method in 3 HPFs was around 10 (9.667), and this value is consistent with the criteria of the number of IgG4+ plasma cells in a core needle biopsy specimen [5, 8]. We also clarified that NOF >10 IgG4+ with the random method was a practical and concise diagnostic tool as a new approach for type 1 AIP.

The optimal cutoff values and accuracies of these parameters were the same and similar, respectively, even in the ROC analyses between type 1 AIP and PDA. This finding indicates that these methods are robust for clinical practice. Furthermore, the bottom line is whether these methods can apply for the use of pancreatic biopsy obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA). Generally, EUS-FNA enables us to distinguish benign from malignant pancreatic mass with around 90 % accuracy [43, 44]. In contrast, the histological diagnosis of AIP by using EUS-FNA was thought to be challenging because of difficulty in collecting an adequate amount of tissue samples for evaluation [4, 44]. However, some reports described that EUS-guided trucut biopsy or EUS-FNA with a 19-gauge needle allowed us to obtain sufficient tissue samples for the diagnosis of AIP [27, 43, 45], although these devices have the potential risk of complications and are difficult in manipulation [46, 47]. Recently, Kanno et al., reported that EUS-FNA using even a 22-gauge needle, with quick motion of the FNA needle and careful sample processing after collection, provided an adequate amount of tissue samples for histological evaluation, and could be used to diagnose >80 % of AIP patients [28]. The article also mentioned that the number of IgG4-positive cells was counted in 10 HPFs selected randomly or all HPFs available, and the average number was calculated [28]. Although the hotspot counting is recommended in a Consensus statement on the pathology of IgG4-related disease [8], it is hard to apply to biopsy specimens. The criteria of mean IgG4+ or NOF >10 IgG4+ with the random method in 3 HPFs in the present study shed light on a simple approach for tissue sampling obtained by EUS-FNA. Usefulness of the random counting method for diagnosis of type 1 AIP should further be validated in prospective large-scale studies.

Finally, we investigated whether the combined criteria of mean IgG4+ and IgG4+/IgG+, which was proposed in the consensus statement on the pathology of IgG4-RD [8] and Japanese comprehensive diagnostic criteria for IgG4-RD [9], provide a more accurate diagnosis of type 1 AIP. Although various cut-off values for mean IgG4+ and IgG4+/IgG+ were evaluated, no combination was superior to the single criterion of mean IgG+ plasma cells or NOF >10 with the random method. This finding illuminated that some cases of CP and PDA showed increased numbers of IgG4+/IgG+ while about 90 % of type 1 AIP cases showed an increased ratio of IgG4+/IgG+, and this finding hindered the improvement of accuracy on the additional IgG4+/IgG+ evaluation. Accurate IgG4+/IgG+ ratios are also sometimes difficult to obtain because of the high background IgG staining [8]. Therefore, we propose that mean IgG4+ >10 or NOF >10 IgG4+ with the random method are the concise and precise methods for the diagnosis of type 1 AIP. For the diagnosis of type 1 AIP, correlation with specific histopathological findings, such as marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration, or storiform fibrosis, is essential, because numerous IgG4+ plasma cells are ubiquitous in diverse non-specific inflammatory conditions and high IgG4+ plasma cells per se do not reliably distinguish type 1 AIP from non-specific conditions [42]. Despite this requirement for other histological features, our new findings allow us to provide a more useful and practical criterion for the diagnosis of type 1 AIP.

The key limitation of the present study was that resected or excisional biopsy specimens of type 1 AIP, CP, and PDA were assembled retrospectively. This was an unfortunate necessity, because type 1 AIP is an uncommon disease and few cases of type 1 AIP have been operated on recently because of improvements in type 1 AIP diagnosis. Recently, Kawano et al., described that infiltrating IgG4+ plasma cells >10/HPF and/or IgG4+/IgG+ (CD138+) >40 % was appropriate for the diagnosis of IgG4-related kidney disease [26]. To clarify these results in other organs such as the bile duct, salivary gland, and lymph node, further investigation is necessary.

In conclusion, we believe that the mean number of IgG4+ plasma cells with a random counting is diagnostic for type 1 AIP. This counting is acceptable even in only 3 HPFs. The additional IgG4+/IgG+ ratio does not increase the accuracy of type 1 AIP identification. Our findings should prove useful in diagnosing patients with type 1 AIP.

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Conflict of interest The authors declare that they have no conflict of interest.

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Screening for IgG4-type anti-nuclear antibodies in IgG4-related disease

Kazuhiro Kiyama, Hajime Yoshifuji*, Tsugumitsu Kandou, Yuji Hosono, Koji Kitagori, Ran Nakashima, Yoshitaka Imura, Naoichiro Yukawa, Koichiro Ohmura, Takao Fujii, Daisuke Kawabata and Tsuneyo Mimori

Abstract

Background: Immunoglobulin (Ig) G4-related disease (IgG4-RD) is characterized by elevated serum IgG4 and infiltration of $IgG4^+$ plasma cells into multiple organs. It is not known whether serum IgG4 is autoreactive in IgG4-RD.

Methods: We measured anti-nuclear antibody (ANA) in 19 IgG4-RD cases, determined IgG subclasses of the ANA, and compared them with those of other systemic autoimmune diseases (systemic lupus erythematosus, Sjögren's syndrome, systemic sclerosis, and polymyositis), using subclass-based ANA test (indirect immunofluorescence).

Results: 58 % of IgG4-RD cases were ANA-positive (cut-off: 1:40). Whereas their subclass of ANA was predominantly IgG2, we observed no IgG4-type ANA. In systemic autoimmune diseases, subclasses of ANA were mostly IgG1, 2, or 3, but IgG4-type ANA was very rarely detected. We also found several patients in whose serum ANA patterns differed among IgG subclasses, probably due to the difference of corresponding autoantigens.

Conclusions: Although IgG4 is highly elevated in sera of IgG4-RD patients, their ANA do not include IgG4 subclass. These results offer new insight into the role of IgG4 and the pathogenesis of IgG4-RD, implying that each IgG subclass tends to cover its own spectrum of antigens, and IgG4 is not preferentially used to make ANA.

Keywords: IgG4-related disease, Systemic autoimmune disease, IgG subclass, Autoantibody, Anti-nuclear antibody

Background

Immunoglobulin (Ig) G4-related disease (IgG4-RD) is a multi-organ disorder characterized by elevated serum IgG4, organ infiltration by $IgG4^+$ plasma cells, hypergammaglobulinemia, and tissue sclerosis [1–4]. Many organs, such as lacrimal gland, salivary gland, eye orbit, lymph node, thyroid gland, lung, pancreas, kidney, retroperitoneum, and prostate can be affected by IgG4-RD. The role of IgG4 in IgG4-RD is not sufficiently understood. Some view IgG4-RD as an allergic disease, because IgG4-RD is often complicated in allergic diseases and serum IgE levels are often high in IgG4-RD. Others see IgG4-RD as an autoimmune disease, because anti-lactoferrin [5] and carbonic anhydrase II [6] antibodies are detected in some of IgG4-related autoimmune pancreatitis cases, and because IgG4-RD cases usually show good responses to glucocorticoid therapies.

At this point, there is no consensus that IgG4-related disease is an autoimmune disorder. To examine whether IgG4 in IgG4-RD is autoreactive, we determined IgG subclasses of serum anti-nuclear antibody (ANA) in IgG4-RD patients and compared them with those in patients with systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), systemic sclerosis (SSc), and polymyositis (PM). Using a subclass-based ANA test that was derived from indirect immunofluorescence (IIF), we investigated how frequently IgG4 was included in ANA in IgG4-RD. We also examined how frequently each IgG subclass was included in ANA in systemic autoimmune diseases.

Methods

Patients

Patients were recruited from Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, Kyoto, Japan. The patients were definitely diagnosed by the 2011 Comprehensive Diagnostic Criteria proposed by the IgG4-RD research team of Ministry of Health, Labour and Welfare (MHLW), Japan [4]: (1) diffuse or

* Correspondence: yossii@kuhp.kyoto-u.ac.jp
Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

localized swelling or mass formation of ≥ 1 organs, (2) elevated serum IgG4 levels ≥135 mg/dL, (3a) fibrosis with remarkable infiltration of lymphocytes and plasma cells, and (3b) $IgG4^+/IgG^+$ plasma cell ratio > 0.4, and > 10 $IgG4^+$ plasma cells in a high-power field. No IgG4-RD patients were considered having SS, Castleman's disease, sarcoidosis, granulomatosis with polyangiitis, or malignant lymphoma. As ANA-positive disease controls, we enrolled 8 SLE patients diagnosed by the 1997 American College of Rheumatology revised criteria [7], 8 SS patients diagnosed by the 1999 revised criteria of MHLW, Japan [8], 4 SSc patients diagnosed by the 1980 American College of Rheumatology criteria [9], and 7 PM patients diagnosed by Bohan and Peter's criteria [10]. All participants provided informed consent in accordance with the Declaration of Helsinki. This study was approved by the Medical Ethics Committee of Graduate School of Medicine and Faculty of Medicine, Kyoto University.

Detection of subclass-specific ANA

We performed subclass-based ANA tests based on the Fluoro-HepANA™ test (Medical & Biological Laboratories, Nagoya, Japan). Briefly, HEp-2 cell-coated slides were incubated with sera, washed with PBS, incubated with FITC-labeled second antibodies, and observed with a fluorescence microscope. Instead of using anti-total human IgG antibody as the second antibody, we used anti-IgG1 (ab50473, Abcam), anti-IgG2 (10122, Alpha Diagnostic Intl.), anti-IgG3 (10123, Alpha Diagnostic Intl.), or anti-IgG4 antibodies (ab99821, Abcam). To detect total-IgG ANA, patients' sera are usually diluted by the ratios starting from 1:40. To detect each IgG-subclass ANA, the sera were not diluted because of relatively low affinities of the second antibodies against subclasses.

Results

ANA positivity of IgG4-RD

Of 19 cases that definitely satisfied the 2011 Comprehensive Diagnostic Criteria for IgG4-RD by MHLW, Japan (Table 1), 14 (74 %) were older than 60 years, and 14 (74 %) were male. Lymph node swellings and retroperitoneal fibrosis were major manifestations. Eleven patients (58 %) were ANA-positive at a cut-off titer of 1:40 (range: 1:40–1:320). The ANA patterns were homogeneous + speckled or speckled in most cases. Although 7 (37 %) were positive for rheumatoid factor and 2 (11 %) were positive for anti-SS-A/Ro antibodies, we confirmed these 9 cases did not fulfill the criteria for rheumatoid arthritis (RA) or SS. No patients were positive for anti-DNA, Sm, or U1-RNP antibodies.

Table 1 Clinical, serological, and histopathological features of IgG4-RD cases

Case	Age	IgG4[a]	IgG[a]	ANA Specific Abs	RF[b]	Clinical manifestations	Biopsy source, $IgG4^+/IgG^+$ cell ratio
1	73	2890	3668	40 (Homo + Spe)	<6	Mikulicz's disease, Prostatitis, LN	Prostate, 0.60
2	76	2210	3632	40 (Spe)	<6	Mikulicz's disease, RPF	Submandibular gl, 0.40
3[c]	79	1460	3669	160 (Homo + Spe) Anti-SS-A^+	<6	Küttner's tumor, IP, IN, RPF, LN	Submandibular gl, 0.73
4	66	1090	2301	40 (Homo + Spe)	30.3	AIP, IN, Renal pseudotumor	Kidney, 0.70
5[c]	73	592	3321	320 (Homo + Spe)	<6	Sialadenitis, IP, IN, RPF, LN	Submandibular gl, 0.43
6	74	389	2184	<40	<6	Retroorbital tumor	Retroorbital tumor, 0.48
7	52	383	1748	<40	<6	Küttner's tumor	Submandibular gl, 0.57
8	70	724	1729	<40	<6	Küttner's tumor, LN	Submandibular gl, 0.40
9	46	675	1617	80 (Homo + Spe)	26.8	Mikulicz's disease	Lachrymal gl, 0.41
10	37	533	1741	<40	<6	Mikulicz's disease	Lachrymal gl, 0.50
11	76	458	1527	<40	<6	AIP, RPF	Retroperitoneal tumor, 0.70
12	62	315	1809	40 (Spe) Anti-SS-A^+	<6	AIP, RPF	Pancreas, 0.43
13	79	1960	2953	40 (Homo + Spe)	65	Orbital tumor, Lung nodule, LN	Orbital tumor, 0.59
14[c]	62	1460	2177	40 (Spe)	23.3	Sialadenitis, Laryngeal tumor, LN	Parotid gl, 0.60 Cervical LN, 0.69
15	65	1050	1811	<40	19.8	Mikulicz's disease, LN	Submandibular LN, 0.80
16	25	1210	2181	<40	<6	Mikulicz's disease, IP, IN, Renal pseudotumor, LN	Minor salivary gl, 0.65
17	55	1510	3116	<40	72.2	Orbital tumor, RPF, Lung nodule, LN	Cervical LN, 0.90
18	61	491	1466	80 (Spe + Granular)	<6	Sialadenitis	Submandibular gl, 0.48
19	78	1470	3762	80 (Homo + Spe)	35	AIP, RPF	Vater's ampulla, 0.48

[a]mg/dL in serum. [b]IU/mL. [c]Shown in Fig. 1

ANA: anti-nuclear antibody; gl: gland; Homo: homogeneous; IN: interstitial nephritis; IP: interstitial pneumonitis; LN: lymph node; RF: rheumatoid factor; RPF: retroperitoneal fibrosis; Spe: speckled

IgG subclasses of ANA in IgG4-RD

We selected 5 IgG4-RD patients robustly ANA-positive with a cut-off titer of 1:80, and examined the IgG subclasses of their ANA. Subclass-based ANA test showed $IgG2^+$ ANA and scant $IgG1^+$ ANA. However, we found no $IgG4^+$ or $IgG3^+$ ANA (Fig. 1, 2). We confirmed that the second antibody against IgG4 worked, using direct immunofluorescence on a lymph node specimen ($IgG4^+/IgG^+$ plasma cell ratio = 0.69) of an IgG4-RD patient (Fig. 1, lower right panel).

IgG subclasses of ANA in systemic autoimmune diseases

We examined IgG subclasses of ANA in systemic autoimmune diseases such as SLE, SSc, SS, and PM (Table 2). The ANA titers ranged from 1:40 to 1:5120 with various patterns. The Subclass-based ANA test detected $IgG1^+$, $IgG2^+$, or $IgG3^+$ ANA in the systemic autoimmune disease cases (Fig. 2, 3). Especially, all cases were $IgG2^+$. However, IgG4 was not detected (Fig. 2, 3), except in a patient with SS who showed IgG4-type ANA with peripheral pattern (Fig. 4).

An exceptional case with IgG4-type ANA

A 79-year-old male with SS showed IgG4-type ANA with peripheral pattern (Fig. 4). Lip biopsy results were compatible with SS, although anti-IgG4 staining was not performed. Anti-SS-A/Ro antibody was positive. We saw no swelling of lacrimal glands, salivary glands, or lymph nodes. This patient did not meet the criteria for IgG4-RD and was not considered to have clinical IgG4-RD.

Difference of ANA patterns among IgG subclasses

In serum from one patient, ANA patterns differed among IgG subclass. Such phenomenon was seen in Fig. 3 and 4.

Discussion

In IgG4-RD patients, we found no $IgG4^+$ ANA, but did detect $IgG1^+$ and $IgG2^+$ ANA (Fig. 1 and 2). We also found $IgG4^+$ ANA was very rare, whereas $IgG1/2/3^+$ ANA were detected in systemic autoimmune diseases (Fig. 2 and 3). Autoantibodies with cytoplasmic patterns in the Fluoro-HepANA™ test are not exact ANA; "anti-cytoplasmic" antibodies—e.g., anti-SS-A/Ro, anti-aminoacyl-tRNA synthetase, and anti-signal recognition particle antibodies—are known in SS and PM. Subclass-based ANA tests found $IgG1/2/3^+$ anti-cytoplasmic antibodies, but not IgG4 (Fig. 2, 3).

$IgG4^+$ ANA is very rare in systemic autoimmune diseases, possibly because serum IgG4/IgG ratios are low, less than 5 %, in these diseases (Table 2). However, $IgG4^+$ ANA was not detected despite high serum IgG4/IgG ratios (43 %) in IgG4-RD. This implies that IgG4 itself is not used to make ANA.

Several studies have investigated ANA subclasses in systemic autoimmune diseases. Zouali et al. reported that in SLE and mixed connective tissue disease, anti-double-stranded DNA (dsDNA) antibody was IgG1/3-dominant, and anti-RNP was IgG2-dominant [11]. Anti-Sm, anti-RNP, and anti-dsDNA in SLE [12], anti-SS-A/Ro and anti-SS-B/La in SS [13], and anti-Scl-70 in SSc [14] are reportedly IgG1-dominant. However, IgG4-type ANA was hardly detected in all the above reports. Rigopoulou et al. examined primary biliary cirrhosis cases, and found that ANA was IgG1/3-dominant but IgG4 was not detected by subclass-based IIF [15]. The reason IgG2-type ANA was remarkably frequent in our study whereas IgG1 and IgG3 were predominant in previous studies might be that second antibody affinities differed between studies. In the subclass-based ANA test, titers cannot be accurately compared between subclasses, as

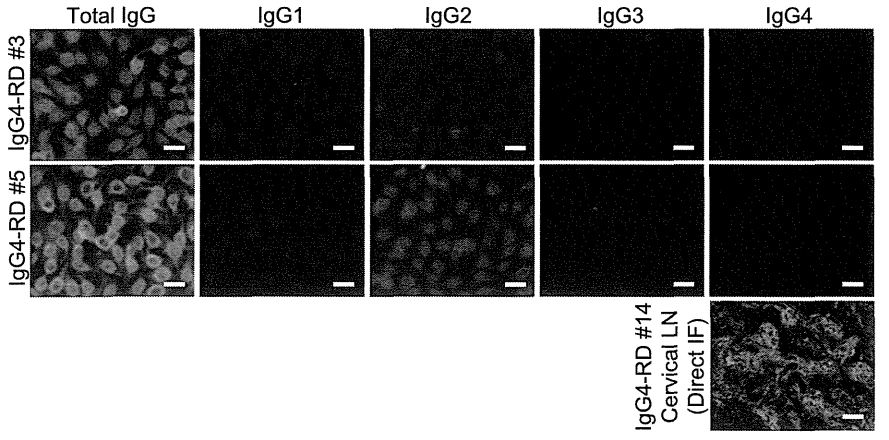

Fig. 1 Subclass-based ANA test in IgG4-RD, showing immunofluorescence microscopy of two typical IgG4-RD cases (IgG4-RD #3 and #5). Lower right panel: We confirmed the second antibody's function by direct immunofluorescence of a lymph node specimen ($IgG4^+/IgG^+$ plasma cell ratio = 0.69) from an IgG4-RD patient (IgG4-RD #14). Bar = 20 μm